Quinone Reductase Activity and the Activation of Mitomycin C Cytotoxicity in the Lens Epithelial Cells

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Distinction Thesis

Medical Dietetics

ABSTRACT

INTRODUCTION: Quinones, naturally found in the human body and environment, are highly reactive molecules that can be metabolized to free radicals and cause oxidative damage to ocular cells, contributing to eye disorders such as cataracts. The phase II detoxifying enzyme NAD(P)H: quinone oxidoreductase 1, also known as quinone reductase (QR), has been found to protect against quinone toxicity. High expression of QR has been detected in the lens epithelial cells. Identifying dietary compounds that promote high QR activity may be critical in preventing oxidative damage to the eye. Additionally, proliferation of residual lens epithelial cells after cataract surgery leads to a common post-operational complication called posterior capsule opacification (PCO). Mitomycin C (MMC) is a cytotoxic drug that is metabolically activated by QR. Because lens epithelial cells have high QR activity, MMC may be useful in treating PCO.

METHODS: Primary dog lens epithelial (DLE) and immortalized human lens epithelial (HLE) cells were cultured. To assess QR expression, the cell lines were treated with the known potent QR inducer β-napthoflavone, and the induced QR activity was assessed using a microtiter plate assay. To assess the role of QR in activating MMC cytotoxicity in DLE cells, MMC was treated in normal DLE cells and cells with dicoumarol-inhibited QR activity; cytotoxicity was assessed with the MTT assay. MMC cytotoxictiy also was compared between DLE and HLE cells and assessed with the crystal violet assay.

RESULTS: Only a 13% increase in QR activity was observed in HLE cells treated with β-napthoflavone (200μM) compared to control cells. QR expression in the primary DLE cell line

was higher than HLE cells, but only a 20% increase in QR activity was observed upon treatment with β-napthoflavone (200μM). As a result, both cell lines were suboptimal for testing QR activation by dietary compounds. Treatment of HLE and DLE cells with MMC (200μM) resulted in 57% and 29% cell death, respectively. Interestingly, MMC-induced cell death appeared to be independent of QR activity levels in both cell lines.

CONCLUSION: Higher QR expression was observed in the primary DLE cell line. The decreased QR expression in the immortalized HLE cells may be a result of immortalization where regulatory and anti-stress proteins have been found to be down-regulated. MMC-induced cytotoxicty in the DLE cell line appears to be independent of QR activity and may be associated with other reductases present in these cells. Identification of these reductase enzymes and their role in mediating MMC-induced cell death may provide additional information regarding the usefulness of this compound in treating PCO in both canines and humans.

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PUBLICATIONS

Research Abstracts and Publications

1. Oonsivilai R, Cheng C, Ningsanond C, Bomser JA, Ferruzzi MG. Induction of quinone reductase activity in murine hepatoma cells by extracts of *Thunbergia Laurifolia Lindl*. FASEB J. 2006;20:A123.3.
2. Liu X, Cheng C, Zorko N, Cronin S, Chen YR, and Zweier JL. Biphasic modulation of vascular nitric oxide catabolism by oxygen. Am J Physiol Heart Circ Physiol. 2004 Dec;287(6):H2421-6.

FIELD OF STUDY

Major Field: Medical Dietetics

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CHAPTER I

INTRODUCTION

Problem Statement

With age, bodily functions begin to deteriorate and operate less efficiently, resulting in greater susceptibility to chronic disease. One theory, the free radical theory of aging, proposes that age-related diseases are due, in part, to increased oxidative damage from free radicals (1). Free radicals are atoms or molecules with one or more unpaired electrons, which makes them unstable. Because these compounds prefer the stable paired-electron state, free radicals are capable of stealing electrons from nearby molecules, damaging critical cellular targets such as DNA, proteins, and lipids (1). As a result, there has been increased interest in identifying the targets and effects of free radicals, as well as compounds that can decrease oxidative damage, such as antioxidants. Antioxidants are molecules that can remain stable as they stop free radical destruction by satisfying their paired electron state. Antioxidants are produced in the body and can also be derived from diet.

One problem that accompanies the aging process is deterioration in ocular health. Even those who have never worn eyeglasses earlier in their life begin wearing reading glasses in order to accommodate their deteriorating eyesight as the lens loses its ability to focus light correctly on the retina. Moreover, many eye disorders accompany deteriorating eye functions, such as age-related macular degeneration, glaucoma, cataracts, and ultimately, blindness (1).

Age-related macular degeneration (AMD) is a disease that causes the central area of the retina to deteriorate, leading to blind spots and blurred or impaired vision. Nearly 1.8 million Americans over the age of forty have advanced AMD, and it is the leading cause of blindness in Caucasians (2). Glaucoma is another disease that leads to the loss of vision through the gradual

degeneration of the optic nerve. About 1.9% (2.2 million) of adults over the age of forty has glaucoma (3). Both diseases contribute to blindness; however, the leading cause of blindness in the world is cataracts. Cataract is a disease that causes clouding in the natural eye lens. About one in six (20.5 million) adults over forty years of age have cataracts, while more than half of Americans over eighty have cataracts (3). One common complication after cataract extraction is posterior capsule opacification (PCO), which develops when residual lens epithelial cells proliferate and cloud the lens capsule. Several costly surgical methods exist, including laser, mechanical scraping, vacuum cleaning, and ultrasonic cleaning, but they are not entirely effective (4). The chemical mitomycin C (MMC) has been investigated for its ability to eradicate the residual epithelial cells and its potential in preventing PCO.

The causes of age-related macular degeneration, glaucoma, and cataracts are not certain; however, the free radical theory of aging may suggest that free radicals contribute to the oxidative stress in the eye. The eye encounters free radicals on a daily basis, whether from cellular metabolism or from UV radiation. In addition, cells in the lens are not renewed; as a result, cells damaged by free radicals remain damaged (5). Similarly, the antioxidant system becomes less efficient with age, which may leave the cells unprotected (1).

Thus, decreasing damage caused by oxidative stress may be essential in preserving the health of the eye. Searching for compounds that can reactivate and/or maintain the antioxidants in the eye may counter the deteriorating antioxidant system as humans age. Increasing intake of antioxidant-rich foods may represent one such strategy. An analysis of scientific research literature regarding one antioxidant mechanism follows.

Review of Literature

The human body is exposed to many foreign compounds (xenobiotics) that can stress and

damage human cells. As a result, the body has developed a system of antioxidant defense mechanisms to fight against the chemicals before they can damage critical cellular components, such as DNA, protein, and lipid. One such mechanism is the phase I and phase II drug metabolizing enzymes. When xenobiotics enter the body, phase I enzymes attach functional groups onto the xenobiotic compounds. This reaction helps phase II enzymes conjugate these xenobiotic compounds into water-soluble products which the body then excretes through the bile or urine (6, 7). The antioxidant of interest, NAD(P)H: quinone oxidoreductase 1 (NQO1), is a phase II enzyme; NQO1 is also known as quinone reductase (QR). This enzyme was first described by Drs. Lars Ernster and Franco Navazio in 1955 when they identified an unusual enzyme in the rat liver that could use both nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) as cofactors (8). Structurally, QR is a flavoprotein consisting of two identical subunits (8). Researchers have discovered many chemical properties of QR: it protects against quinone toxicity, maintains the antioxidant potential of endogenous antioxidants, and stabilizes the p53 anti-tumor protein (9). The protective nature of QR was first described when QR levels increased after being exposed to low doses of carcinogenic agents (10). QR was identified as a phase II enzyme, and it was postulated that this enzyme and others may prevent cancer at the tumor-initiation stage by detoxifying potential carcinogens (11).

Quinones are highly reactive molecules that are found in both the human body (i.e. estrogen) and the environment (i.e. cigarette smoke, vehicle exhaust). Once exposed to certain enzymes (i.e. P450 reductase), quinones can readily form semiquinone intermediates, which, once reorganized, become free radicals (9). Chemicals in this state are very destructive to cells as they attempt to reinstate their paired-electron state. Semiquinones are further destructive in

that they can produce more quinones, thus leading to the formation of additional reactive oxygen species, such as superoxide and hydroxyl radicals. Because QR can reduce quinones by two electrons, instead of one, this enzyme produces more stable hydroquinones (Fig. 1), which are less destructive and more easily excreted from the body (9).

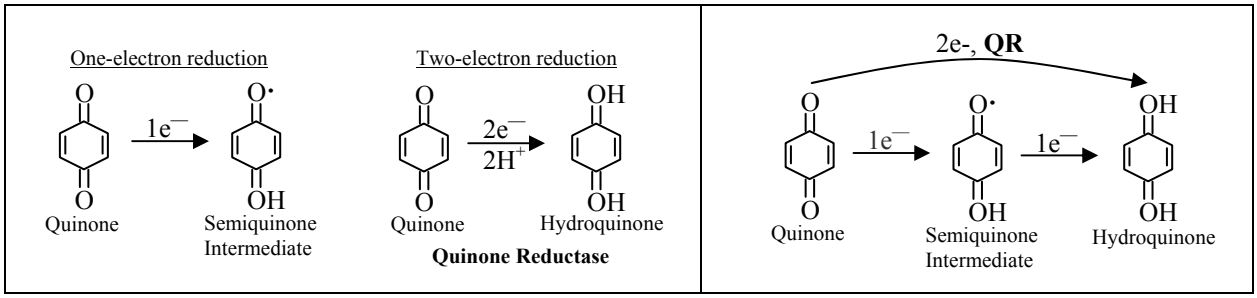

Figure 1. One-electron vs. two-electron enzymatic reductions of QR (12, 9)

QR is also responsible, in part, for maintaining the antioxidant potential of various compounds; two examples are ubiquinone (coenzyme Q) and α-tocopherol-quinone (vitamin E). Both antioxidants contain substrates for QR. Ubiquinones are distributed to the cell membrane when oxidative stress is detected, and QR reduces the ubiquinones into uniquinol, which protect against phospholipid destruction (Fig. 2). Similarly, QR reduces α-tocopherol-quinone to its

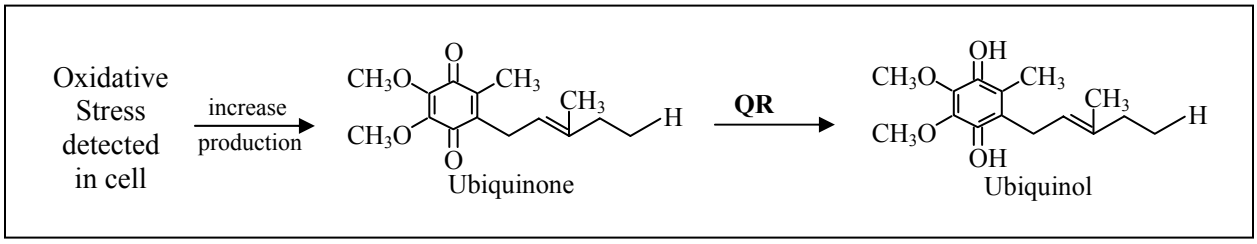

Figure 2. Role of QR in reducing ubiquinone to ubiquinol

potent antioxidant state, α-tocopherol-hydroquinone (Fig. 3), when cells detect free radical presence (9).

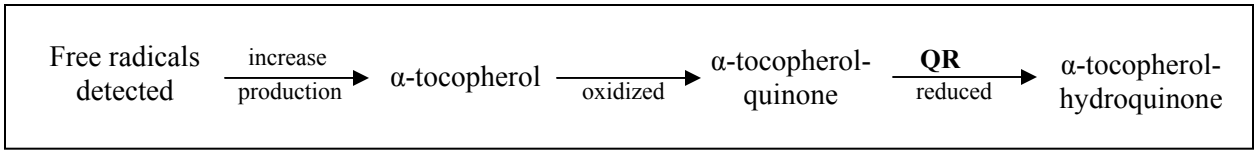

Figure 3. Role of QR in reducing α-tocopherol (8)

Lastly, QR has also been found to stabilize p53, a protein that inhibits tumor growth through increases in apoptosis. The protein is relatively unstable and can be degraded by various enzymes. Researchers have found that QR prevents p53 degradation; however, the exact mechanism by which QR stabilizes p53 protein requires further examination (9).

Studies have located the QR enzyme in numerous epithelial cells throughout the human body, including lung, breast, colon, vascular endothelium, adipocytes, cornea, lens, retina, optic nerve, and nerve fibers (9). Interestingly, this enzyme can be activated by a number of chemically diverse dietary compounds, including β-carotene, ascorbic acid, phenols, azo dyes, coumarins, sulfur compounds, flavones, indoles, retinoids, tocopherols, and selenium (13, 14). The majority of studies examining dietary activation of QR have used liver as the enzyme source, while only a limited number have examined activation of this enzyme in other tissues (15, 16).

In one study, Gao and Talalay (17) demonstrated that the dietary compound sulforaphane found in cruciferous vegetables protect the retinal pigment epithelial cells against photooxidative damage. The lens of the eye is especially vulnerable to oxidative stress because of its inability to renew cells (18). In addition, it is hypothesized that oxidative damage contributes to the development and progression of many age-related macular degenerative diseases (17). Studies have found that quinones contribute to oxidative damage in the eye, leading to diseases such as cataracts and retinal degeneration (19, 20). Qian and Shichi (19) have found that quinone metabolites injected into lens cells increased intracellular Ca^{2+}, leading to opacity in the eye. In an immunohistochemistry study by Siegel and Ross (21), high levels of QR expression were observed in the corneal and lens epithelium. The relatively high expression of QR in the lens suggests that this enzyme may provide protection against oxidative stress in this tissue.

Additionally, there is interest in the ability of the anti-proliferative drug MMC to treat the

post-operative cataract complication PCO. Interestingly, the cytotoxic effects of MMC is activated by QR or related reductases. The high levels of QR in the lens epithelium facilitate this activation. PCO results from the proliferation of remnant epithelial cells after cataract surgery, and research has found that MMC can reduce these remnant cells. MMC is a naturally occurring antibiotic that is derived from the microorganism *Streptomyces caspitosus* and causes cytotoxicity by damaging the DNA. This cytotoxic capability is activated by reducing MMC via one electron yielding a semiquinone or via two electrons yielding a hydroquinone. Several enzymes are capable of this reduction, including the two-electron reductase QR (22). In a study investigating the mechanism of MMC-induced cell death on mouse lens epithelial cells, Park et al (23) found that dicoumarol reduced MMC-induced cell death by 80%. Since dicoumarol is a known inhibitor of QR, this confirms the relationship between MMC and QR. However, the inability of dicoumarol to completely inhibit MMC cytotoxicity also confirms that MMC is activated by reductases other than QR.

Studies outlined in this paper are designed to identify dietary compounds that can activate QR lens epithelial cells, as well as investigate the role of QR in activating MMC in both primary canine and immortalized human lens epithelial cells.

Objectives

We hypothesize that dietary compounds previously shown to activate QR in the liver will also activate this enzyme in the lens. In addition, we expect that increased QR activity in the lens cells will provide protection against oxidative insult. We anticipate that results from these studies will provide novel insight into the role of QR in the lens cells and how dietary modulation of this enzyme may protect these tissues from oxidative insults.

The dietary compounds we plan to test are:

- β-carotene (vitamin A)—carrots, sweet potato
- Lutein—corn, egg yolk, green vegetables and fruits
- Lycopene—red tomatoes, watermelon, papaya, pink grapefruit
- Ascorbic acid (vitamin C)—fruits (citrus, berries), vegetables (broccoli, spinach)
- Tocopherol (vitamin E)—cereals, nuts, sunflower seeds, cooking oils
- Thiamin (vitamin B1)—wheat bran, nuts, lean pork, beans, sesame seeds, cereals
- Riboflavin (vitamin B2)—dairy, eggs, mushrooms, green vegetables, cereals
- Sulphoraphane—broccoli

Additionally, we anticipate that QR will activate MMC in the lens epithelial cell lines resulting in cellular toxicity. Furthermore, we hypothesize that MMC-induced cytotoxicity will be reduced upon inhibition of QR with dicoumarol.

CHAPTER II

MATERIALS AND METHODS

In this study, we plan to investigate QR expression as well as the role of QR in activating MMC cytotoxicity in primary DLE and immortalized HLE cells.

Materials

FAD, NADP, glucose-6-phosphate, baker's yeast glucose-6-phosphate dehydrogenase, menadione, digitonin, dicoumarol, β-napthoflavone, MTT, and Mitomycin C (MMC) were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbeco's Modified Eagle Medium (DMEM) and fetal bovine serum were obtained from GIBCO. Dog lens epithelial (DLE) cells were harvested from euthanized dogs from the Franklin County Humane Society.

Methods

Tissue culture

Human lens epithelial (HLE) cells were grown in T-75 flasks with 10mL DMEM supplemented with 10% fetal bovine serum (FBS). Dog lens epithelial (DLE) cells were grown in Laminin-coated T-25 flasks with 4mL DMEM supplemented with 10% FBS and penicillin/streptomycin (to prevent contamination).

Cell preparation

HLE cells were seeded in plates ranging in sizes from 12, 24, and 96 wells to 60mm and 100mm individual dishes at a concentration of 100,000 to 1,500,000 cells/well in 200µL to 10mL of DMEM supplemented with 10% FBS. The cells were incubated for 24 hours in a humidified incubator at 37°C to allow the cells to attach. The medium was aspirated and refed with 200µL to 10mL of DMEM supplemented with 10% FBS. The chemical compounds to be tested were mixed with the medium at the appropriate concentration. The first lane was assigned

as the control lane and was left untreated; the wells were filled with 200µL to 10mL of DMEM supplemented with 10% FBS. The cells were incubated for 24 to 72 hours depending on the experimental procedure.

DLE cells were seeded in 48-well plates, 96-well plates, and 60mm individual dishes at a concentration of 30,000 to 1,000,000 cells/well in 200µL to 3mL of DMEM supplemented with 10% FBS and penicillin/streptomycin. The cells were incubated for 24 hours in a humidified incubator at 37°C to allow the cells to attach. The medium was aspirated and refed with 200µL to 3mL of serum-free DMEM. The chemical compounds to be tested were mixed with the medium at the appropriate concentration. The first lane was assigned as the control lane and was left untreated; the wells were filled with 200µL to 3mL of serum-free DMEM. The cells were incubated for 24 hours.

Quinone reductase screening assay

The activation of QR was assessed using a modified quinone reductase assay based on the method of Prochaska et al (24). Stock solutions of the following chemicals were prepared beforehand and stored for aliquoting later when the assay solutions were made: 50mL of 0.5M Tris-Cl (pH 7.4), 1mL of 7.5 mM FAD, 10mL of 150mM glucose-6-phosphate, 1mL of 50mM NADP, 1mL of 50mM menadione, 50mL of 2mM EDTA, and 10mL of 5mM K_2PO_4 with 0.5% DMSO. For the assay, the following stock solution was prepared: 1.25mL of 0.5M Tris-Cl (pH 7.4), 16.67mg of bovine serum albumin, 166.7µL of 1% Tween-20, 16.67µL of 7.5mM FAD, 166.7µL of 150mM glucose-6-phosphate, 15µL of 50mM NADP, 50U of baker's yeast glucose-6-phosphate dehydrogenase, 7.5mg of MTT, and distilled in water to a final volume of 25mL. 0.16µL of 50mM menadione dissolved in acetonitrile was added just before the mixture was added to the 96-well plates.

After the cells were treated for 24 hours, the cells were lysed. The lysis solution was made with 0.8% digitonin and 2mM EDTA. The media were aspirated and 50µL of the lysis solution was added to each well; the cells were incubated for 10 minutes at 37°C. The plates were then placed on an orbital shaker and agitated for 10 minutes at 25°C. Next, 200µL of the assay stock solution described above was added to each well, and a blue color should develop. After 5 minutes, the reaction was arrested by adding 50µL per well of a solution containing 0.3M dicoumarol in 0.5% DMSO and 5mM K_2PO_4 (pH 7.4). For the control lane, the wells only contained the assay stock solution. Finally, the plates were scanned in a plate reader at 610nm.

Quinone reductase catalyzes NADPH to reduce menadione to menadiol; when menadiol reduces MTT, a blue color is generated. The level of QR activation can then be quantified by reading the absorbance of the blue color using the plate reader. The results of QR activity will be reported and compared as the ratio of the absorbance readings from the treated concentration wells to the control lane within the same 96-well plates. These ratios will be used to analyze the effect of the dietary compound concentration on QR activity and to compare the effectiveness of QR activation between the various tested dietary compounds.

Crystal Violet

To account for variations in cell growth caused by different treatment chemicals, the crystal violet assay was utilized to assess cell viability. The assay assesses the approximate number of cells in each well by staining the total cellular mass which is proportional to the cellular number. The cellular medium was aspirated from the wells and 150µl of 0.2% crystal violet in 2% ethanol was added to each well and incubated for 10 minutes. The crystal violet dye was removed by submerging the plate in distilled water several times. Once air dried, 50µl of 0.5% SDS in 50% ethanol was added and incubated for 45 minutes to 1 hour. The plate was then

read with a spectrophotometer at 620nm.

MTT Assay

Cell death by MMC was assessed with the MTT assay. Three milligrams of MTT dissolved in 1mL of PBS was sterile filtered and 15µL of the filtered solution was added directly into the medium in each well; the plate was incubated for about 4 hours. The medium was aspirated, and 150µL of 0.04 mol/L HCl in isopropanol was added to each well. The crystals were allowed to dissolve by incubating the plate at room temperature for at least 5 minutes. The plate was then read with a spectrophotometer at 595nm.

CHAPTER III

RESULTS AND DISCUSSION

Results

QR and dietary compound experimentation

We were unable to test QR activation by the dietary compound listed in the "Objectives."

HLE and QR expression

In 96-well plates seeded with 50,000 and 100,000 cells/well and serial diluted with 2µM β-napthoflavone as the high concentration, no blue color was observed when the arresting solution was added after 5 minutes. The spectrophotometer reading yielded no observable patterns (Fig. 4). When the cellular density was increased to 1,000,000 cells/well seeded in a 24-

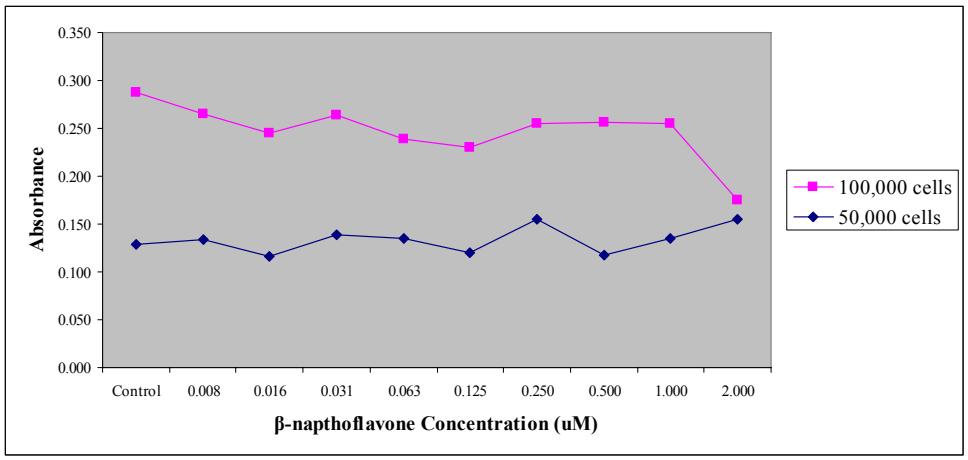

Figure 4. Mean absorbances of HLE treated with 2µM β-napthoflavone for 24 hours for two different cell densities.

well plate and the high β-napthoflavone concentration increased to 20µM, the bluish-color developed slowly after the reaction mixture was added for 20 to 25 minutes. The spectrophotometer still yielded no significant pattern of absorbance change.

When the cell density was returned to 100,000 cells/well seeded in a 96-well plate and the high β-napthoflavone concentration increased to 100µM for 72 hours, the bluish-color change remained slow. After approximately 30 minutes, there was no clear color gradient and

the color between the control and high concentration lanes were similar. The spectrophotometer reading yielded an increase in induced QR activity, but only a 13% increase was observed between the control and the high concentration lanes (Fig. 5).

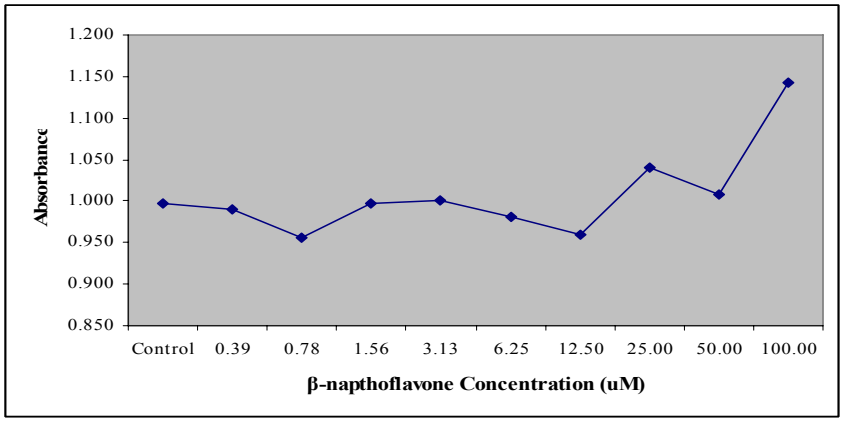

Figure 5. Mean absorbance of HLE treated with β-napthoflavone for 72 hours.

DLE and QR expression

In the 96-well plate seeded with 75,000 cells/well, 3 lanes were reserved for testing dicoumarol inhibition of QR activity. The arresting solution was added instead of the reaction mixture and the spectrophotometer yielded very low absorbance readings (0.040-0.072). The remaining 7 lanes tested QR induction by β-napthoflavone. Bluish-color development occurred rapidly and yielded a mean absorbance reading of 2.254 for the high concentration (100µM) after 1 minute; a 20% increase between the control and high concentration was observed (Fig. 6).

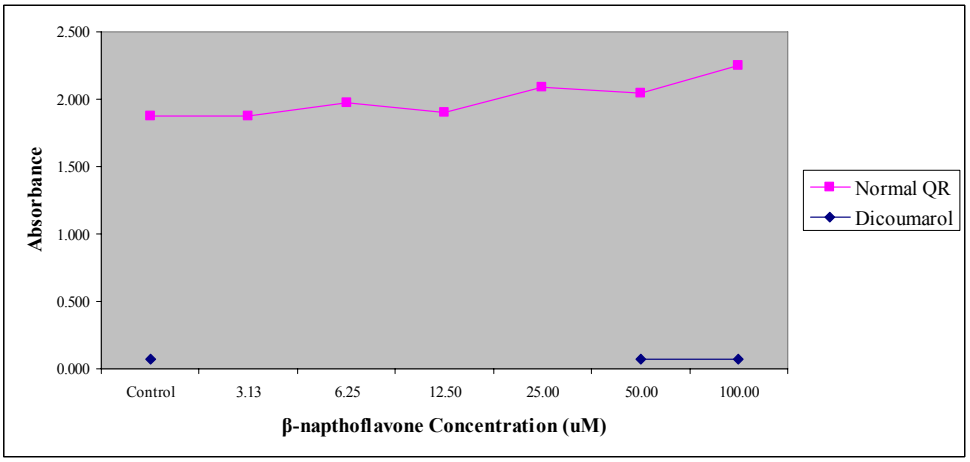

Figure 6. Mean final absorbance readings of QR induced activity for adding arresting solution first and for normal QR assay procedure

DLE with MMC

In a laminin-coated 48-well plate seeded with 30,000 cells/well, the cells treated with 100µM dicoumarol for 30 minutes and then serial diluted with MMC for 1 hour yielded a 33.9% decrease in cell concentration between the control and high concentration (200µg/mL). For the cells without dicoumarol treatment, a 41.9% decrease was observed (Fig. 7).

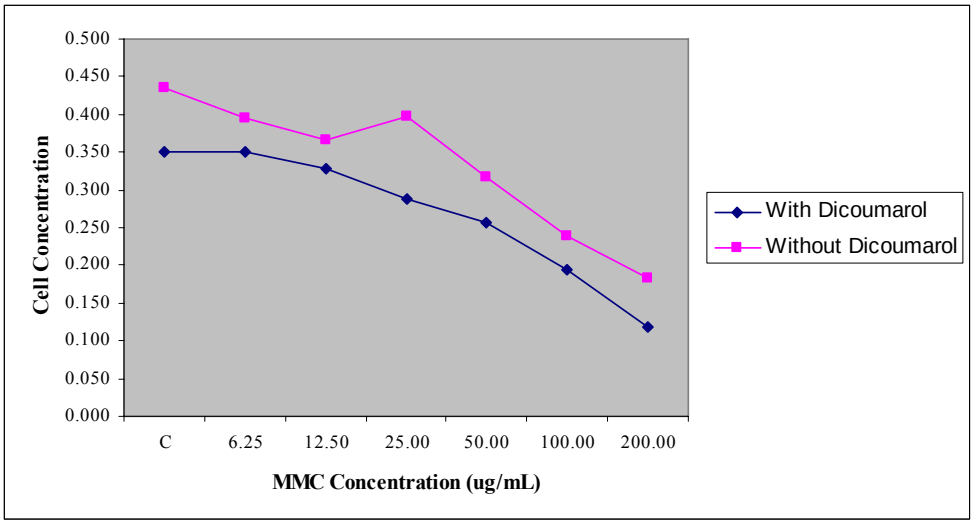

Figure 7. Cell Toxicity by MMC with and without dicoumarol treatment

HLE and DLE with MMC

Twelve 60mm dishes were seeded with 200,000 cells/dish—6 dishes with HLE cells and 6 with DLE cells—and allowed to attach and proliferate for 3 days. The mean percentage of cell death observed between the control and 200µg/mL of MMC was 57% and 29% for HLE and DLE, respectively (Fig. 7).

	Number of cells (10^6)		
	Control	MMC (200µg/mL)	Cell Death
HLE	2.742	1.186	57%
DLE	2.040	1.444	29%

Figure 7. Mean cell number in control and MMC treatment and percent cell death caused by MMC

The results for the DLE cells may be skewed due to a high concentration of cells in each well; overconfluency may have increased the actual cytotoxicity caused by MMC.

Discussion

Unfortunately, both the HLE and DLE cell lines were unsuitable for testing QR response to the dietary compounds. The slow QR assay color development and low absorbance reading in the HLE cells treated with the known potent QR inducer β-napthoflavone prevented the possibility of accurately identifying QR response to dietary compound treatment. Similarly, the high QR expression in the DLE cells and the small difference (20%) in QR activation between the high concentration β-napthoflavone treatment (100μM) and the control created a suboptimal environment for testing QR response to less potent dietary compounds. As a result, we did not test QR response to dietary compounds.

The delayed bluish-color development and low absorbance reading for the HLE cells suggested that QR expression is low in these cell lines. Even after increasing the number of cells, β-napthoflavone concentration, and treatment time, color development remained slow. We hypothesized that this may be due to the immortalization of the HLE cell line. Immortalized cells are derived by transfecting primary cells with tumor viruses and/or growth genes (25). Research suggests that immortalized cells have decreased expression of apoptotic proteins (e.g. p53 regulatory proteins) and increased expression of telmorase which maintains cell life (26). Ibaraki et al (27) elucidated that immortalized HLE cells have lower expressions of α and β crystallins, lens structural proteins found to exhibit anti-stress capabilities (28). Since immortalization affects protein expressions that defend against stress and promote cellular life span, we decided to test QR expression in the primary cell line DLE.

There was a distinct difference in the QR assay reaction between the HLE and DLE. The bluish-color development caused by the reduced MTT from the QR-reduced menadiol occurred almost immediately and the absorbance readings were significantly higher. The high absorbance

reading in the control suggested that QR expression is naturally high in DLE cells. With a known potent QR inducer, a high concentration of 100μM β-napthoflavone resulted in only a 20% increase from the control. This small difference suggests that the cell line may not be appropriate for testing dietary compounds that may be less potent in inducing QR. Instead, we decided to use the high QR expression in DLE cells to test MMC.

Research has found that QR can activate MMC. There are studies that investigate treating PCO with MMC in humans. The MMC and PCO relationship has been explored in various animal lens epithelial lines but not in canines. Investigations in human lens epithelial cells have revealed that MMC helps reduce but not completely eliminate PCO (29); however, complications from MMC leakage to other ocular regions exists, including scleral ulceration, corneal perforation, scarring of conjunctiva and cornea, formation of retrocorneal membrane, iritis, secondary glaucoma, and cataract (4, 23). In our investigations, we found that dicoumarol does not inhibit MMC cytotoxicity as effectively as seen in the mouse lens epithelial cells (23). When MMC activation by QR was compared between a low QR expression and a high QR expression cell line, greater cell death was not observed in the high QR expression as was hypothesized. These results suggest that MMC is not specific to QR and that QR may not be the primary mechanism of MMC activation in the DLE.

Furthermore, the decreased expression of α and β crystallins in the immortalized HLE cells may confirm the greater cell death observed in the HLE cells treated with MMC versus the DLE cells. Since DLE is a primary cell line, there may be greater defense mechanisms against cytotoxic elements. Contrarily, the decreased expression of p53 regulatory proteins that promotes apoptosis and increased expression of telomerase that maintains cellular life in immortalized cells would suggest that HLE cells would yield lower cell death. With results

contrary to these expectations, the greater HLE cell death may confirm that MMC-induced cytotoxicity is not related to regulatory proteins.

Although further research is required, these results suggest that MMC may not be an optimal treatment options for PCO in canine or humans.

Limitations

Several limitations existed which may have affected the observed results. Cell counts were estimated using a hemocytometer which involved counting only a small volume of cells to estimate the total number in the total volume. The DLE cells required Laminin-coated flasks; and the Laminin may have had interactions with the chemicals in the assays that may have skewed the final results.

All the assays—QR, crystal violet, and MTT—contained inaccuracies. The freshness and the accuracy in the measurement of the chemicals may have affected the effectiveness of the assays. For the QR assay, the manual addition of the mixtures may have affected the spectrophotometer readings since time lapsed between the additions in the first and last lanes. Also, the chemical reaction in the assay itself between the NADPH, menadiol, and MTT and the ability of the lysis buffer to completely lyse the cells may have influenced the ability to determine the actual level of induced QR activity. For the crystal violet assay, unattached cells were lost while rinsing, which skewed the final cell number count. And for the MTT assay, the cell number was determined by staining the mitochondria in the cells; if the cells did not have mitochondria, an accurate cell count could not be obtained.

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